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ECOLOGY AND MOLECULAR GENETIC STUDIES OF MARINE BACTERIA

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Sequencing of the chitinase operon of *V. vulnificus* is complete. In vitro transcription/translation analyses indicate the synthesis of a structural protein 100kD in size. Analyses with deletion mutants of the original 'chitinase' clone are being used to determine translation initiation and termination sites. Results indicate that chitin hydrolysis by *V. vulnificus* is accomplished via a glucosaminidase rather than, or in addition to, the classical two enzyme (chitinase and chitobiase) system. The rapid isolation method of prokaryotic DNA from aquatic habitats has been successfully used to recover plasmid DNA's, stable RNA's and DNA samples from hydrothermal vent fluids. The inclusion of a glass fiber prefiltration step has facilitated sampling of increased volumes of water. Optimization of the sensitivity of DNA probes to detect low copy number genes in bacteria from environmental samples has been accomplished. Purified 'black smoker DNA' was ligated with vector DNA and eight clones have been isolated. DNA sequencing of the cloned DNA is proceeding in order to compare these sequences with known archaeobacterial DNA sequences. Cloned DNA will serve

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for the preparation of DNA probes to be used to analyze future isolates of 'black smoker DNA'. 5S rRNA sequences from V. mediterranei 50T, V. furnissii ATCC #35016, V. orientalis ATCC #33934, V. campbellii ATCC #25920, V. hollisae ATCC #33564, and V. costicola ATCC #33508 have been determined. 5S rRNA has been isolated and purified from V. fluvialis ATCC #33809, V. neoclistes ATCC #14636, V. nigrapulchritudo ATCC #17043 and Alteromonas hanedai ATCC #33224 in order that their sequences be determined. Preliminary DNA/DNA homology data suggest that V. ordallii and V. anuillarum; V. fluvialis and V. furnissii; and V. harveyi and V. carchariae may represent only three species. The relative binding ratios obtained indicate that some species may be more correctly assigned to a genus other than Vibrio. (AW)



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Project Goals:

The objectives of the research are to gain an understanding of the ecology, systematics, and molecular genetics of marine bacteria, crucial to the development of marine biotechnology and its applications. Sub-projects are to (i) clone and sequence ecologically relevant genes from members of the genus Vibrio, including deep-sea species, (ii) examine, by means of DNA homology, relatedness between members of the genus Vibrio; (iii) characterize structure-function relationships between 5S rRNA and the 50S ribosomal subunit to elucidate the role of 5S rRNAs and (iv) clone and sequence genes from deep-sea bacteria.

Recent Accomplishments

The characterization of relevant genes as well as the tracking of genetically engineered organisms (GEMS) often necessitates the ability to monitor the presence of these genes in a habitat. When studying aquatic habitats, the process of monitoring a specific nucleic acid sequence can be complicated by the species diversity of the habitat, the relative abundance of sequences in total DNA pool, and the volume of the habitat. Gene probes (DNA/DNA, DNA/RNA) can be used as a sensitive tool to detect genes even when organisms cannot be grown on artificial media, or if the gene is not expressed (cryptic). In addition, it is possible to differentiate between genes that are active and inactive by first probing for the gene (DNA) and subsequently probing the transcript (RNA). Given enough probes directed towards environmentally relevant gene sequences, it may be

possible to characterize quickly a habitat by presence or absence of groups of organisms and their relative activities. We have approached this rather broad and complex problem through three specific areas of research; i) the isolation and characterization of an environmentally important bacterial operon (the chitinase operon of Vibrio vulnificus) as a model system, ii) the development of a method to facilitate the isolation of total procaryotic DNA from water samples, and iii) the development of a system to enhance the sensitivity of nucleic acid probes. Substantial progress have been achieved in these areas.

The sequence of the chitinase "operon" of Vibrio vulnificus has essentially been completed. It was previously believed that there were two open reading frames in the sequence data, but recent data reveal only one long open reading frame. The sequence data are borne out by the results of in vitro transcription and translation analyses which indicate one translation product of nearly 100 kDa from the cloned DNA. Further transcription and translation experiments with deletion mutants of the original clone have been completed and will be used to determine translation initiation and termination sites.

These results suggest that chitin degradation in Vibrio vulnificus is accomplished via a glucosaminidase rather than, or in addition to, the classical two enzyme (chitinase and chitobiase) system. Glucosaminidases, sometimes referred to exochitinases, degrade the chitin polymer by processively removing NAG residues from the non-reducing end. True chitinases, whether endo-or exo-lytic, hydrolyse chitin to

chitobiose which is then split by chitobiase to form NAG residues.

Further progress has been made in the development of a method for the rapid isolation of total procaryotic nucleic acids from aquatic samples has been reported (C. Somerville et al., 1988. REGEM 1 Abstract #40, Sterivex method). Cells are first concentrated by ultrafiltration, lysed and nucleic acids partially purified within the filter housing. The method has been shown to be effective for recovery of chromosomal DNA of sufficient purity to be detected by DNA probes. With very little modification, this method may also be used for recovery of plasmid DNA and stable RNAs.

An important application is the direct recovery of nucleic acids from microorganisms in extreme environments, where the ability to culture the microbiota is difficult, if not impossible. For example, this method has been successfully applied to samples of hydrothermal vent fluids..

The application of Sterivex method has been used for the rapid isolation of nucleic acids from aquatic samples of black smoker hydrothermal vent fluid from the Juan de Fuca and 21°N vents. Glass fiber prefilters were not used. Amounts as large as 16ng of DNA per ml of vent fluid were recovered. Other experiments employing the method, using Chesapeake Bay water samples, have shown an average recovery of 1 ng DNA per 10^6 cells, suggesting that the vent fluids may contain as many as 1.6×10^7 cells ml^{-1} . In addition, the purest smoker fluid (i.e. relatively unmixed with ambient sea water) did not yield

detectable levels of DNA until the sample concentrate was subjected to lysis by a French Press, suggesting that, in the natural environment, this DNA is contained within a protective structure not labile to standard enzymatic lysis.

The purified DNA from the pure smoker fluid samples which remained after quantification was pooled and used in a cloning experiment. Eight clones of 'black smoker DNA' were produced. Due to sheering of the DNA during French Press lysis, the size of the cloned DNA was small, the largest insert being approximately 350 bp. The cloned DNA is being sequenced and the sequences will be compared with archebacterial sequences already available in our on-line data base. The smaller inserts will be used to construct probes to use with black smoker samples obtained in future sample collecting.

The significant advantages of the Sterivex isolation method include elimination of the need for culturing, relatively large sample volume, and the recovery of stable RNA as well as DNA from a single sample. However, sample volume is limited by the capacity of the filters used. Sampling volume could be increased through the development of a prefiltration system for use with Sterivex filters.

In a recent report by Fuhrman et al. (1988) the use of glass fiber prefilters (Gelman type A/E, 47 mm) was shown to be effective for filtering several liters of seawater to collect picoplankton for direct extraction of nucleic acids. We have tested this type of prefilter with the Sterivex (TM) filters using samples of Chesapeake Bay water, and found that while the

volume of water passed through the Sterivex (TM) unit can be increased, with consequent increase in nucleic acid recovery, they also trap some of the bacteria in the sample, and prevent them from reaching the collection filter. When the prefilter is placed in front of the sterivex unit and changed each time a liter of water is filtered, a six to ten-fold increase in the volume of sample concentrated on the sterivex unit is achieved. This is reflected in an increased recovery of both DNA and rRNA species from the samples.

When the prefilters are homogenized and Acridine Orange Direct Counts (AODC) done to enumerate cells on the prefilters, from 8 to 13% of the sample population (confirmed by AODC) is retained on the prefilter. This may well represent a specific sub-population in the sample, such as large bacteria or bacteria predominately attached to particulates in the sample. The type A/E prefilters, however, are useful for increasing the size of the sample which can be concentrated onto one sterivex unit. At sites where it was possible to filter only several hundred ml through a single sterivex unit, we are now able to filter several liters. In studies where it is important to recover large quantities of nucleic acids directly from a water sample, such as cloning and probing for rare determinants, benefits of the glass fiber prefilters outweigh the disadvantage of retention of a minor component of the population.

Given the species diversity and volume of aquatic habitats, it is crucial to increase the sensitivity of gene probes for specific nucleic acid sequences. A method for generating

increased signal from a given probe, while retaining high specificity, has been developed in our laboratory (Somerville et al., 1988. REGEM 1, Abstract #70). DNA polymerase is used to add radiolabeled nucleotides to a probe-target heteroduplex, thereby increasing signal at sites specified by the probe. The potential for non-specific polymerization priming from free 3' hydroxyl residues is reduced by replacing the 3' termini with dideoxy residues. The method does not alter the concentration of target sequences in the environmental sample. A series of field trials will be necessary to determine the efficacy of the method for environmental samples.

In work related to our primary objective for this project, genes responsible for bioluminescence in Vibrio cholera biotype albensis have been cloned into the suicide vector pEcoR251. Escherichia coli transformed with this construct acquires the bioluminescent phenotype without the requirement for addition of aldehyde, suggesting that all of the genes required for light production are present.

To determine relatedness between V. cholerae biotype albensis and two other bioluminescent species, Vibrio harveyi and Vibrio fischeri, the genes which code for the luciferase enzyme (luxAB) are being sequenced. Restriction analysis shows that these genes are contained on a 9-kb fragment. A detailed restriction map of the cloned fragment has been obtained. Southern blots of restriction digests of the cloned DNA has been probed with the luxAB genes from V. fischeri to determine the location of the luciferase genes on the cloned fragment.

Sequencing is now in progress on fragments contained in the region showing homology.

The restriction digest information was also useful in subcloning the luxAB genes from V. cholerae biotype albensis cloned fragment. The fragment was subcloned into three different reading frames of pUC plasmids (pUC8, pUC9, pUC19) to obtain the genes which code for the luciferase enzyme in the reading frame required for expression. Screening of the subclones has not yielded a clone which is luminescent upon addition of aldehyde substrate. DNA probes prepared from the V. fischeri luciferase a- and b-subunit genes have been used in colony hybridizations. Nonluminescent strains of V. cholerae have been found to possess similar regions in their genomes.

The 5S rRNA sequence of Vibrio mediterranei 50T has been determined. Analysis of the sequence suggests that V. mediterranei belongs to the Gamma-3 subdivision of the Rhodobacteria and is clearly related to the genus Listonella (MacDonell and Colwell, 1985). Comparison of the V. mediterranei 5S rRNA sequence to those of other Listonella spp. shows that it is most closely related to L. tubiashii ATCC 19105 (99.2%; Pillidge and Colwell, 1988) and L. pelagius ATCC 25916 (98.3%; MacDonell and Colwell, 1985). Homology of greater than 90% was also observed with L. aestuarianus ATCC 3504B, L. damsela ATCC 33539, L. ordalli ATCC 33509 and L. anguillarum ATCC 19264.

We are currently determining the sequences of 5s rRNA molecules of the remaining type strains of the family Vibrionaceae, and of genera closely related to Vibrio. During

the last six months the sequences of the 5s rRNA molecules of the following species have been determined after 3' end labelling by ^{32}P :

Vibrio furnissii (ATCC #35016)

Vibrio orientalis (ATCC #33934)

Vibrio campbellii (ATCC #25920)

Vibrio hollisae (ATCC #33564)

Vibrio costicola (ATCC #33508)

In addition, 5s rRNA has been isolated and purified from V. fluvialis (ATCC #33809), V. neocistes (ATCC #14636), V. nigrapulchritudo (ATCC #27043) and Alteromonas hanedai (ATCC #33224). We anticipate that these species will be 3' end labelled and the sequences determined early in the new year. Thereafter, sequences of all the above mentioned isolated will be confirmed after 5' end labelling.

DNA/DNA homology studies have been performed in order to examine relatedness among members of the genus Vibrio. DNA/DNA homologies for Vibrio harveyi, V. carchariae, and V. parahaemolyticus against nine species of the family Vibrionaceae have been determined. The relative binding ratios (RBRs) and melting temperatures (T_m) of hybrids have been determined under both relaxed (60°C) and stringent (75°C) conditions. Initial results indicated that the RBRs for intergeneric hybridizations range between 3.0 - 100%, with a drop in T_m of the hybrids ranging between 0.9°C - 17.6°C. and the RBRs for intrageneric hybridizations range between 8.0 - 9.1%, with a drop in T_m of the hybrids ranging between 14.3 - 17.1°C. These results suggest a

need to redefine the assignment of some species presently within the genus Vibrio.

DNA/DNA hybridizations among Vibrio and related species will be entered. For example, six species (V. ordalii, V. parahaemolyticus, V. anguillarum, V. fluvialis, V. fischeri and Photobacterium angustum) have been hybridized at low stringency (60°C) against all 31 test species and the RBRs of the hybrids have been determined. Preliminary examination of the data indicate that V. ordalii and V. anguillarum are closely related: 77.4% and 68.0% RBRs with a 1.8°C and 1.4°C drop in T_m s of the hybrids from the homologous controls, respectively. In addition, V. fluvialis and V. furnisii show a high RBR of 97.3% with a 1.1°C drop in T_m . These results suggest that both these pairs (V. ordalii/V. anguillarum and V. fluvialis/V. furnisii) may be reassigned to two single species following further comparison with numerical taxonomy and 5S ribosomal RNA sequence data. V. parahemolyticus may represent a "median" or "mode" species of the genus Vibrio since it shows a moderate degree of homology to many of the test species.

A more detailed analysis of the V. harveyi/V. carchariae relationship has been performed under both relaxed and stringent conditions. Relative binding ratios of greater than 92% were obtained with a decrease in the T_m of less than 1°C. These results are in agreement with results of biochemical tests and suggest that these two strains are of the same species. However, 5s rRNA sequences indicate phylogenetic differences between the two species. Homology studies further indicate that the V.

harveyi chromosome may be significantly larger than that of V. carchariae, possibly a function of gene duplication in V. harveyi. Reassignment of V. carchariae as a strain of V. harveyi is therefore suggested.

Future Work:

1. Disable the chitinase gene within Vibrio vulnificus using marker exchange techniques and assay for the loss of chitin-clearing phenotype.
2. Ascertain the nonluminescent phenotype of V. cholerae strains by assaying for low-level light production through the use of a liquid scintillation counter. Sequencing will continue to obtain the complete nucleotide sequence of the luxAB genes in V. cholerae biotype albensis. Prokaryotic directed in vitro transcription/translation analysis will be used to determine the number and molecular weights of the gene products encoded by the lux operon of V. cholerae biotype albensis.
3. Complete sequencing of V. fluvialis, V. neocistis, V. nigrapulchritudo and Alteromonas hanedae 5s rRNA molecules. The 5s rRNA's of the following ATCC type strains will be isolated and sequenced:
 - V. adaptatus (ATCC #19263)
 - V. cyclocites (ATCC #14635)
 - V. logei (ATCC #29985)
 - V. splendidus (ATCC #33125)
4. Completion of the DNA:DNA hybridization matrix for the remaining 25 Vibrio and related organisms. Analyses of the relationship among the test species will be carried out using a

three dimensional plot to compare hybridization, numerical taxonomy and 5s rRNA sequence data in order to establish a phylogeny for the family Vibrionaceae.

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